EVALUATION OF A RICIN VACCINE CANDIDATE FOR HUMAN TOXICITY USING AN *IN VITRO* VASCULAR LEAK MODEL

Aimee Porter*[1], Martha L. Hale[2], Amanda Pace[3], John-Michael Rosario[1], Luis DaSilva[1]
[1]Center for Aerobiological Sciences, [2]Toxinology and [3]Pathology Divisions,
United States Army Medical Research Institute of Infectious Diseases
Fort Detrick, MD 21702

ABSTRACT

Inhaling lethal doses of ricin (RT) causes pulmonary edema and results in death. In lower concentrations, ricin minimally causes tissue damage to the respiratory tract and lungs, which can incapacitate the individuals exposed to this toxin. A genetically derived ricin A chain vaccine candidate (RTA 33/198) is available that lacks the cytotoxic N-glycosidase activity. RTA 33/198 was previously shown to be protective in animal studies against an aerosolized RT challenge. The current study evaluated if this ricin vaccine is safe for use in humans by using an *in vitro* vascular leak assay. Incubating human endothelial cells with the vaccine at concentrations ranging from 0.6 to 9 μM had no considerable toxic effects in measures of transendothelial electrical resistance for up to 24 hr. Cytotoxicity due to exposure of 9 μM ricin A chain in culture was apparent by changes in electrical resistance as early as 2 hr. Electron micrographs of endothelial cells exposed for 6 hr to 9 μM of the vaccine had reduced cytotoxicity when compared to cells treated with the ricin holotoxin. Because the binding of ricin to the endothelial cell surface may suggest the occurrence of vascular leak, we performed flow cytometry of the endothelial cells exposed to either RTA, RTA 33/198 vaccine candidate, or the ricin holotoxin to detect binding of the toxin to the cell. Our results indicated significant binding of the ricin holotoxin, and possibly a slight binding of ricin A chain. However, there was no binding by the ricin vaccine RTA 33/198. This human vascular leak model provides an adequate *in vitro* testing system to evaluate toxicities intrinsic to vaccines and can even be used for pre-testing therapeutics to biowarfare agents, reducing the need of animals in scientific research. In this specific study, the model successfully demonstrated the reduced toxicity of the lead ricin vaccine when compared to the toxicity caused by holotoxin and to ricin A chain.

1. INTRODUCTION

Ricin is a toxin derived from the castor bean plant, *Ricinus commmunis.* This toxin consists of two polypeptide chains, the A chain and the B chain. The A chain carries the enzymatic activity responsible for depurinating the ribosomal RNA, which blocks protein synthesis and ultimately causes cell death (J Biol Chem. 262:8128-30, 1987; Med Leg J. 48:51-62, 1980). The B chain has the lectin property that allows the toxin to bind to complex galactosides on cell-surface carbohydrates and be internalized (J Biol Chem. 258:5933-7, 1983). The severity which ricin affects human health depends on dosage and route of exposure. With the Kcat for the N-glycosidase enzymatic reaction approximately 1800 min-1, one molecule is sufficient to kill a cell (FEBS Lett. 195:1-8, 1986). In animal studies, inhaling small air particles of ricin can cause respiratory distress, pulmonary lesions, and alveolar inflammation (Vet Pathol. 33:296-302, 1996). Attempts to make a vaccine have proven difficult due to receptor promiscuity of ricin and this toxin's ability to affect a vital cell survival function, protein synthesis. Existing countermeasures may prevent lethality but not the appearance of secondary complications such as pulmonary edema and incapacitation.

In addition to its enzymatic activity, the deglycosylated form of the ricin A chain contains peptide motifs that bind to human endothelial cells and cause damage to human skin grafted onto SCID immuno-incompetent mice (PNAS 96:3957-62, 1999). Several reports suggest that the motifs are responsible for the vascular leak syndrome (VLS), a common side effect observed after ricin A chain immunotherapy (Exp Cell Res. 206:227-34, 1993; Exp Cell Res. 258:417-24, 2000). Receptors for these peptide motifs have been found on human endothelial cells, but not on rodent endothelial cells (PNAS 96:3957-62, 1999). This becomes important because the murine model is commonly used to assess toxicity, and, to date, VLS has not been detected in this model (Immunopharmacology 37:117-32, 1997; Semin Cell Biol. 2:47-58, 1991).

The risk for an intramuscularly administered ricin vaccine to cause VLS is lower than for immunotoxins given intravenously. However we still need to show that VLS is not a complication after inoculating the vaccine into humans. Recent efforts showed that vaccine candidates using recombinant ricin A chains devoid of enzymatic activity protected mice from ricin (Protein Eng. Des. Sel., 17(4):391-7. Epub 2004 Jun 08). RTA 33/198 developed at USAMRIID provides protection in animal studies and no longer inhibits translation as tested by a cell-free translation assay (Protein Eng. Des. Sel.,

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**00 DEC 2004**
2. REPORT TYPE	**N/A**
3. DATES COVERED	-
4. TITLE AND SUBTITLE	**Evaluation Of A Ricin Vaccine Candidate For Human Toxicity Using An In Vitro Vascular Leak Model**
5a. CONTRACT NUMBER	
5b. GRANT NUMBER	
5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)	
5d. PROJECT NUMBER	
5e. TASK NUMBER	
5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	**Center for Aerobiological Sciences, Toxinology and Pathology Divisions, United States Army Medical Research Institute of Infectious Diseases Fort Detrick, MD 2170**
8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	
10. SPONSOR/MONITOR'S ACRONYM(S)	
11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT	**Approved for public release, distribution unlimited**
13. SUPPLEMENTARY NOTES	**See also ADM001736, Proceedings for the Army Science Conference (24th) Held on 29 November - 2 December 2005 in Orlando, Florida., The original document contains color images.**
14. ABSTRACT	
15. SUBJECT TERMS	

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**4**	

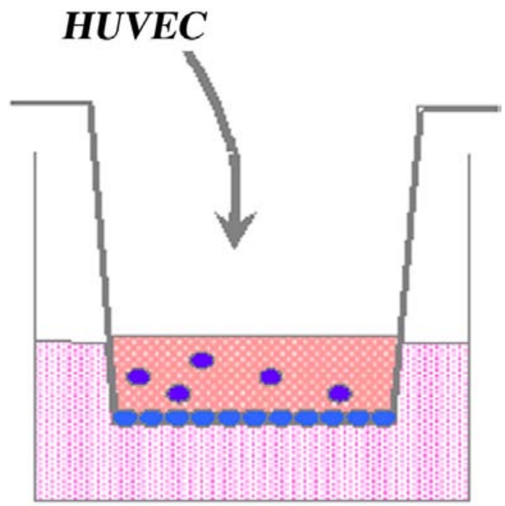
Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

17(4):391-7. Epub 2004 Jun 08; Hale, person, comm.). However, this vaccine candidate contains the peptide motif previously shown to bind to human vascular endothelial cells and therefore, there is a possibility that RTA 33/198, while enzymatically inactive, could cause VLS in humans. A safe and effective vaccine against ricin intoxication must include studies confirming that the vaccine will not induce VLS in humans. The *in vitro* vascular leak assay can use human primary endothelial cells to reveal if there are any toxicities in the ricin vaccine.

2. FIGURES AND TABLES

Figure 1. Structure of ricin A chain showing N-Terminal domain = green, C-terminal domain = purple, RTA loop structure = red, epitope-binding site = orange (Protein Sci. 2:429-35, 1993). In the RTA 33/198 vaccine, only amino acids 1-33 and 44-198, located at the N-terminal domain, are present.

A.



B.

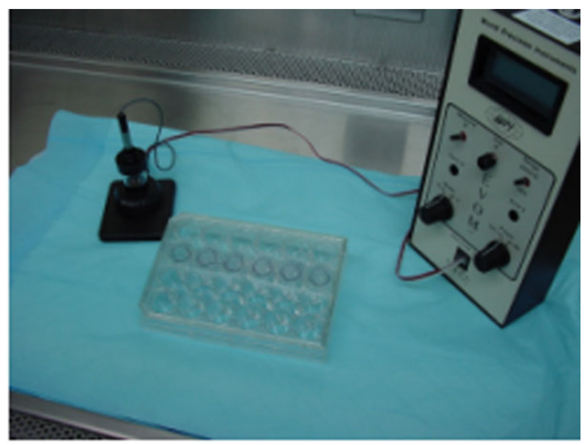


Figure 2. Measurement of transendothelial electrical resistance. (A) In the *in vitro* vascular leak model, human primary endothelial cells (HUVEC) were grown to confluency on the surface of a cell environment insert immersed in cell growth medium in a 24-well plate. Confluency of the cell monolayer was determined by measuring the transendothelial electrical resistance (TEER) every other day, placing the insert containing the cell in a electrical chamber containing cell growth medium. (B) The chamber had one electrode on each side of the insert. An electric current was passed through the insert and the electrical resistance was measured by the Endohm voltmeter.

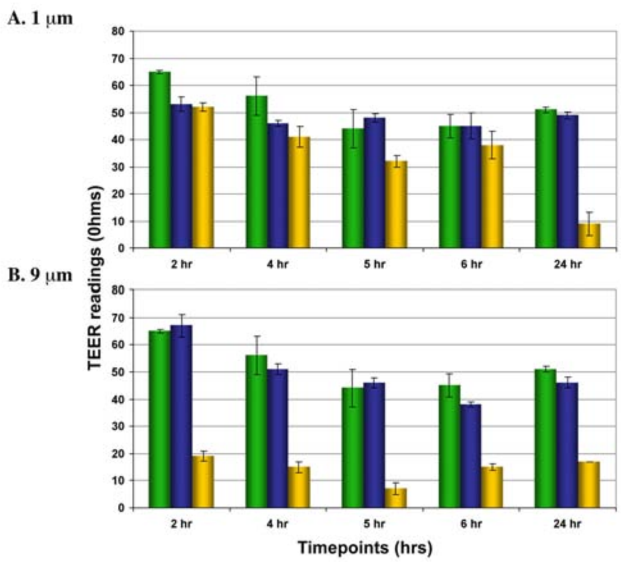


Figure 3. The toxicity of the ricin vaccine candidate RTA 33/198 (blue) was measured by its ability to rupture the integrity of the endothelial monolayer of HUVEC in an *in vitro* vascular leak model. Measurements of TEER were performed over a wide range of concentrations (0.6– 9 µM), and compared with the responses to ricin A chain (RTA, yellow) with a incubation period of up to 24 hr. (A) In this representative experiment, at 1 µM, only RTA showed considerable toxicity after exposure as evidenced by a drop in TEER at 24 hr. At this concentration, the vaccine RTA 33/198 (blue) showed no prolonged toxic effect for up to 24 hr exposure. (B) At the highest concentration tested (9 µM), RTA (yellow) was toxic as early as 2 hr after exposure. No prolonged toxic effect was observed for the vaccine (blue). The green bars represent unexposed HUVEC.

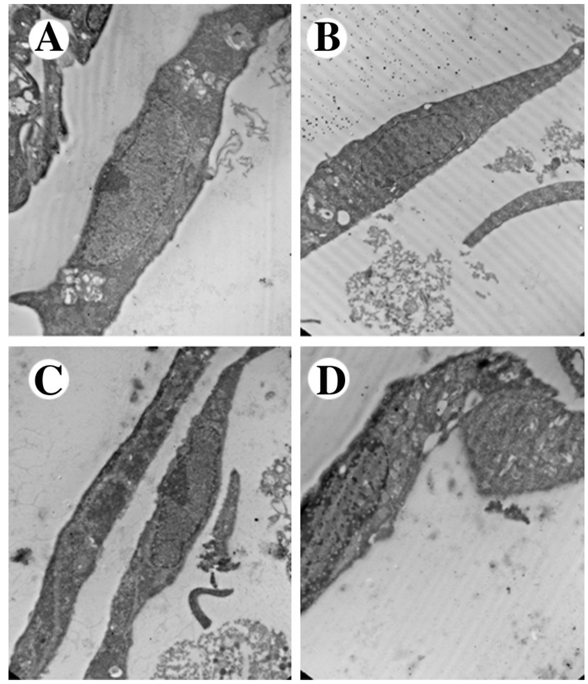


Figure 4. Transmission electron micrographs (TEMs) of activated HUVEC. Endothelial cells (HUVEC) were exposed to either (A) medium alone or to 9 µM of (B) RTA, (C) RTA 33/198, and (D) ricin holotoxin for 6 hr. TEMs were prepared from pelleted, treated HUVEC. The recovered cells, treated with either medium alone, RTA, or RTA 33/198 shared no considerable difference in the cell structure. However, cells treated with the holotoxin appeared more vacuolated and showed that the chromatin was starting to condense, indicative of cell damage.

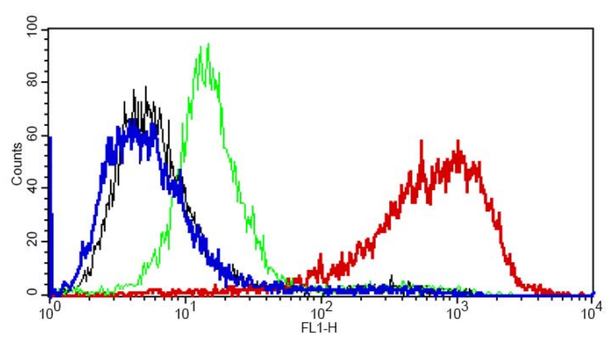


Figure 5. Flow cytometry of RTA or RTA 33/198-exposed HUVEC were performed and compared to ricin holotoxin to determine whether the ricin vaccine RTA 33/198 bound to the surface of HUVEC. Cell-surface binding of ricin is thought to account for vascular leak cytotoxicity effects. In this representative experiment, the cells were treated with a hyperimmune serum to the deglycosylated form of RTA after incubation with either 0.6 µM of RTA (green), ricin holotoxin (red), or RTA 33/198 (blue). The black line represents the fluorescence

profile when only secondary antibody was added to the cells, with no ricin or RTA primary antibody. Significant binding of ricin holotoxin to the HUVEC was observed (red). A slight shift caused by the binding of RTA (green) was also noted. However, no binding was detected by the ricin vaccine RTA 33/198 (blue).

3. CONCLUSION

Our results presented here show that RTA 33/198, at a minimum, had reduced cytotoxicity to endothelial cells *in vitro*, as compared to RTA, and therefore may provide an efficacious vaccine against inhaled ricin. These investigations also provide methodology to determine whether biowarfare agents or vaccines will damage endothelial cells, possibly resulting in vascular leak syndrome.

ACKNOWLEDGEMENTS

We thank Mrs. Kathy Khuel, LTC JoLynne Raymond, and SPC Ray Casteline, for their technical expertise with the electron micrographs.